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HEALTH SCIENCES

Cupuaçu extract protects the kidneys of diabetic rats by modulating Nrf2/NF-κB p65 and iNOS

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Abstract: Diabetes is characterized by increased levels of oxidative stress. Its suggested that extract of cupuaçu could improve the antioxidant system in diabetes. The aim was to evaluate the effect of EC on Nrf2/NF-κB p65 in normal and diabetic rats. Male, adult Wistar rats (9-week-old) were distributed in 4 groups: control (CTL) and diabetic (DM) who received water; CTLEC and DMEC who received 1 mL/day of EC (1 g/mL), via gavage for 8 consecutive weeks. The diabetes was inducted with a single intravenous dose of 45 mg/kg streptozotocin. Glycemia and body weight were measured at the beginning and end of the protocol, and the renal tissue was analyzed by Western blot for SOD-1, SOD-2, CAT, GSSG, Nrf2, NF-κB p65, iNOS and 3-NT. Glycemia was reduced in DMEC vs. DM after 8 weeks of EC treatment. There was no difference in body weight of DMEC vs. DM; however, DMEC vs. DM presented increased levels of CAT and Nrf2, with a significant reduction of NF-κB p65, iNOS and 3-NT. Therefore, we suggest that EC could be utilized as a complementary therapy to ameliorate the antioxidant profile via Nrf2 and to delay the evolution of diabetic complications in renal tissue by inflammatory pathway inhibition.

Key words: Cupuaçu, diabetic kidney, iNOS, NF-κB p65, Nrf2, 3-NT.

INTRODUCTION

Diabetes mellitus compromise several organs, especially the kidney, leading to diabetic nephropathy (DN), contributing to micro- and macroalbuminuria until the significant loss of renal function. Hyperglycemia stimulates the production of reactive oxygen species (Gross et al. 2005) and reactive nitrogen species (RNS), promoting oxidative and/or nitrosative stresses, and both ROS and RNS can cause damage to lipids, proteins and DNA (Fiorentino et al. 2013, Mastrocola et al. 2005). Overproduction of ROS can activate nuclear factor kappa B (NF-κB), which is involved in the encoding of several pro-inflammatory genes as well as nitric oxide synthase (NOS), responsible for the synthesis of nitric oxide (NO), on harmful effects, such as cellular injury and inflammation (Taylor et al. 1998). NO can even participate in the S-nitrosation process, in which the nitration of tyrosine residues through peroxynitrite (produced by reaction between NO and superoxide anion) generates 3-nitrotyrosine (3-NT), modifying the function of several proteins (Matough et al. 2012). Oxidative stress also reduces the antioxidant capacity of superoxide dismutase (SOD), catalase (CAT), glutathione peroxidase (GPx) and glutathione reductase (Gross et al. 2005) enzymes. These antioxidant proteins may be enhanced by the action of nuclear factor erythroid 2-related factor 2 (Nrf2) (Colak et al. 2005, Goth 2008, Goth et al. 2001, Knapik-Kordecka et al. 2007).

Polyphenols are able to chelate metals and eliminate ROS/RNS. Within this context, flavonoids are potential antioxidants present in

fruits and vegetables, having associations with cardioprotection, reduction of blood pressure, and improvement of the antioxidant profile (Engler 2004). It was observed that Theobroma grandiflorum has an important amount of quercetin, which belongs to the flavonoid group, contributing to the improvement of lipid profile and antioxidant capacity in diabetic rats, and cupuaçu was able to increase insulin sensitivity (Yang et al. 2003, Jalil et al. 2008). A previous study in our laboratory showed that daily consumption of cupuaçu pulp, in the concentration of 1 g/mL, which is equivalent to one cup (200 mL/day) of cupuaçu juice for a person, no added sugar improved the control of nitrosative stress and ROS production and reduced inflammatory factors (Punaro et al. 2019). Furthermore, the cupuaçu pulp is rich in fiber, which can be us and can be used in other products, giving it a different flavor.

Considering that diabetes is a chronic disease with a high level of oxidative stress and that diabetic patients have reduced antioxidant capacity; the aim was to evaluate the effect of EC on antioxidant and inflammatory profiles in streptozotocin (STZ) induced diabetic rats.

MATERIALS AND METHODS

Animals

Male Wistar rats, 8 weeks of age, weighing ± 250 g, were obtained from Central Animal Housing of "Escola Paulista de Medicina" (São Paulo, SP, Brazil). All protocols were approved by the Ethics Committee in Research of Universidade Federal de São Paulo (protocol Nº 7381030317). The animals were maintained in the animal housing of the Nephrology Division at a temperature of 22 ± 1 ºC and a light–dark cycle of 12/12 h, beginning at 6:00 am. The animals were given free access to standard chow (Nuvital CR-1, Nuvilab, PR, Brazil) and water. The rats were allocated into four groups: control group (CTL); control group that received cupuaçu (CTLEC); diabetic rats (DM) and diabetic rats that received cupuaçu (DMEC), n = 5 for each group.

Induction of type 1 diabetes

After seven days of adaptation, 9-week-old animals received a single intravenous administration of STZ 45 mg/kg dissolved in 0.1 M cold citrate buffer, pH 4.5. The control rats received only citrate buffer. After 72 h of induction, glycemia was measured in blood samples collected from the tail vein, and the values were determined using a glucometer. Diabetes was defined in this study with glycemia ≥ 200 mg/dL (mimicking the same value in humans) after 3 h of fasting, according to the protocol previously established in our laboratory (Punaro et al. 2014).

Preparation of EC

The frozen pulp of cupuaçu (200 g) obtained from "Sítio do Bello Frutas Nativas ME" (São Paulo, SP, Brazil), protected from light, was diluted in 200 mL of filtered water (1 g/mL), homogenized, sifted and separated in aliquots in sterile tubes in a daily amount of use, avoiding repeated thawing of the product, and stored in a freezer at -20ºC until use. According to information that includes packaging of the product purchased for the present study, 100 g of native and frozen cupuaçu pulp contains 72 Kcal, 14.7 g of carbohydrates, 0.0017 g of protein, 1.6 g of lipids and 0.5 g of dietary fiber.

Drug administration

The CTLEC and DMEC groups received EC starting on the 4th day after diabetes induction. The administration was carried out for 8 consecutive weeks via gavage at a dose of 1 mL/day (1 g/mL). The other groups, CTL and DM, received water as vehicle. Glycemia (mg/dL) was measured

using a glucometer before and after 4 and 8 weeks of treatment, and body weight (g) was checked during the same period. At the end of the protocol, the animals were euthanized with anesthetic (90 mg/kg of ketamine hydrochloride and 18 mg/kg of xylazine hydrochloride at, intraperitoneally), followed by an incision of the diaphragm and the kidneys were collected for analysis.

Western blot analysis

The protein levels of SOD-1, SOD-2, CAT, oxidized glutathione (GSSG), Nrf2, NF-κB p65, inducible nitric oxide synthase (iNOS) and 3-NT were assessed in renal cortex samples (removed from both kidneys) and the protein concentration was quantified by the Bradford method. Briefly, 80 µg of protein were separated in a 12% polyacrylamide gel and transferred to a nitrocellulose membrane. Subsequently, the membranes were probed against SOD-1 (1:500; sc-11407), SOD-2 (1:1,000; sc-30080), CAT (1:2,000; C 0979), GSSG (1:2,000; MAB5310), Nrf2 (1:500; sc-722), NF-κB p65 (1:500; sc-372), iNOS (1:200; sc-651) or 3-NT (1:500; sc-32757) followed by anti-mouse or anti-rabbit secondary antibodies, 1:1,000 or 1:5,000. The bands were analyzed by gel documentation Alliance 4.7 Uvitec (Cambridge, Cambs, UK). The relative protein content was normalized using actin (1:1,000; sc-1615). This assay was repeated at least four times for each protein.

Statistical analysis

The results are expressed as the mean and standard error of the media (SEM). First, the Kolmogorov-Smirnov normality test was utilized, and the differences among the four groups were examined for statistical significance using one-way analysis of variance (ANOVA), followed by the Newman-Keuls multiple comparison posttest for parametric data (glycemia, body weight, Nrf2 and 3-NT) or Kruskal-Wallis followed by Dunn's multiple comparison posttest for nonparametric data (SOD-1, SOD-2, CAT, GSSG, NF-κB p65 and iNOS). Values were considered statistically significant when $P<0.05$; tests were performed on the GraphPad Prism 5.0 program (GraphPad Software Inc, San Diego, USA).

RESULTS

Effect of EC treatment on glycemia levels and body weight in rats

Glycemia (mg/dL) of the diabetic group was significantly elevated in relation to the respective control (322.0 ± 39.1 vs. 112.3 ± 1.2, $p < 0.05$), as well as DMEC vs. CTLEC groups (335.0 ± 23.7 vs. 115.0 ± 5.2, $p < 0.05$), before EC treatment; however, DM vs. DMEC did not show significant differences (Fig. 1).

After 4 weeks of EC treatment, the CTL and CTLEC groups remained unchanged in this parameter. There were significant differences in DM vs. CTL (385.0 ± 41.8 vs. 112.0 ± 2.9, $p < 0.05$) and between DMEC vs. CTLEC (315.8 ± 72.9 vs. 111.5 ± 6.5, $p < 0.05$), with no significant difference between DM vs. DMEC (Fig. 1).

After 8 weeks of EC, there were significant differences in DM vs. CTL (378.8 ± 50.8 vs. 116.0 ± 3.3, $p < 0.05$) and between DMEC vs. CTLEC (325.0 ± 48.6 vs. 114.8 ± 2.6, $p < 0.05$) with no significant reduction in DMEC vs. DM, Fig. 1.

Before EC treatment, the body weight (g) of the groups had approximate values (CTL, 277.5 ± 8.1; CTLEC, 289.0 ± 7.9; DM, 245.6 ± 12.1 and DMEC, 279.1 ± 12.8), with no significant difference among them, as seen in Fig. 1.

After 4 weeks of EC treatment, the DM group presented a significant decrease in body weight (g) in relation to the CTL (267.2 ± 16.1 vs. 319.2 ± 10.1, $p < 0.05$), while DMEC presented a non-significant decrease in relation to the CTLEC

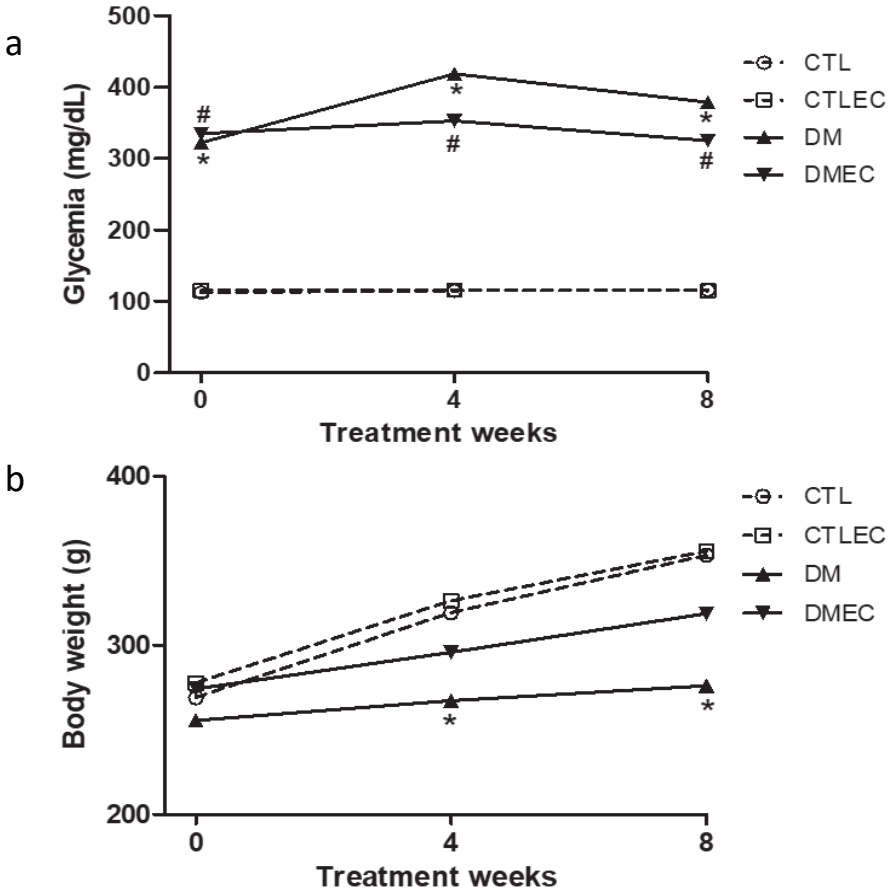

Figure 1. Glycemia (a) and body weight (b) of normal and diabetic rats before and after EC treatment. EC: extract of cupuaçu. CTL: control that received water. CTLEC: control that received EC. DM: diabetic rats that received water. DMEC: diabetic rats that received EC. n = 5 per group. ANOVA followed by Newman-Keuls Multiple Comparison post-test. $p < 0.05$: *vs. CTL; #vs. CTLEC.

(295.9 ± 19.9 vs. 326.2 ± 6.2), as well as DMEC vs. DM (Fig. 1).

After 8 weeks of EC treatment, the DM group maintained a significant decrease in body weight (g) in relation to the CTL (276.1 ± 12.5 vs. 353.2 ± 10.6, $p < 0.05$), while DMEC maintained a non-significant decrease in relation to the CTLEC (318.8 ± 21.8 vs. 355.5 ± 10.4), similar to DMEC vs. DM (Fig. 1).

Effect of EC treatment on antioxidant profile and redox balance in rats

SOD-1 (intensity ratio) had a significant reduction in DM vs. CTL (0.93 ± 0.06 vs. 1.83 ± 0.09, $p < 0.05$) and DMEC vs. CTLEC (0.97 ± 0.06 vs. 1.88 ± 0.09, $p < 0.05$), with no difference between the diabetic groups (Fig. 2).

SOD-2 (intensity ratio) had a significant reduction in DM vs. CTL (1.07 ± 0.05 vs. 1.39 ± 0.12, $p < 0.05$) and DMEC vs. CTLEC (0.99 ± 0.05 vs. 1.59 ± 0.08, $p < 0.05$), with no difference between the diabetic groups (Fig. 2).

CAT (intensity ratio) had a significant reduction in DM vs. CTL (1.02 ± 0.05 vs. 1.49 ± 0.12, $p < 0.05$), without a difference between DMEC vs. CTLEC (1.34 ± 0.06 vs. 1.60 ± 0.12). However, there was a significant increase in DMEC vs. DM ($p < 0.05$).

GSSH (intensity ratio) was increased significantly in DM vs. CTL (1.40 ± 0.05 vs. 0.81 ± 1.12, $p < 0.05$) and in DMEC vs. CTLEC (1.28 ± 0.04 vs. 0.89 ± 0.06, $p < 0.05$), with no difference between the diabetic groups (Fig. 2).

Nrf2 (intensity ratio) was significantly reduced in DM vs. CTL (0.55 ± 0.04 vs. 1.75 ± 0.11,

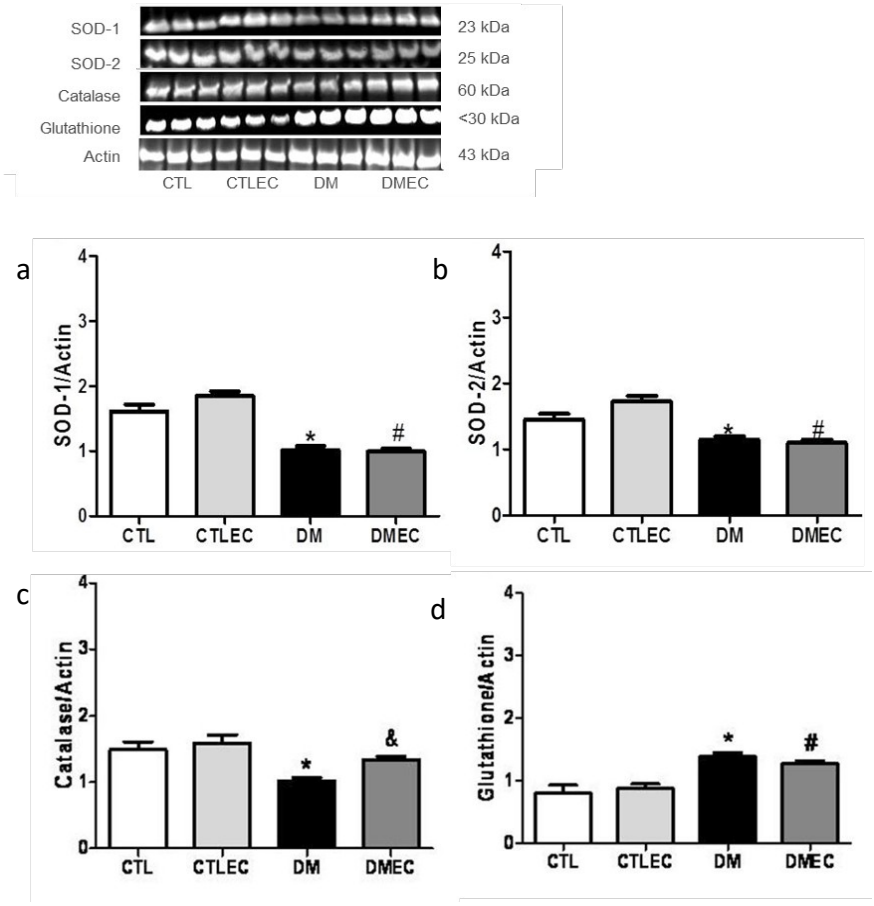

Figure 2. Analysis of SOD-1 (a), SOD-2 (b), Catalase (c) and glutathione (d) in renal tissue of normal and diabetic rats after EC treatment. EC: extract of cupuaçu. SOD: superoxide dismutase. CTL: control that received water. CTLEC: control that received EC. DM: diabetic rats that received water. DMEC: diabetic rats that received EC. n = 5 per group. Kruskal-Wallis test followed by Dunn's multiple comparison post-test. p < 0.05: *vs. CTL; #vs. CTLEC; &vs. DM.

p < 0.05) and DMEC vs. CTLEC (1.16 ± 0.03 vs. 1.89 ± 0.11, p < 0.05). However, it was significantly increased in DMEC vs. DM, Fig. 3.

NF-κB p65 (intensity ratio) had a significant increase in DM vs. CTL (2.53 ± 0.16 vs. 0.72 ± 0.03, p < 0.05), with no significant difference between DMEC vs. CTLEC (1.37 ± 0.07 vs. 0.68 ± 0.03); DMEC was significantly reduced vs. DM, Fig. 3.

There was a significant increase in iNOS (intensity ratio) in DM vs. CTL (1.48 ± 0.06 vs. 0.60 ± 0.04, p < 0.05), as well between DMEC vs. CTLEC (0.96 ± 0.04 vs. 0.68 ± 0.01, p < 0.05; however, DMEC was significantly reduced vs. DM, Fig. 3.

3-NT (intensity ratio) was increased significantly in DM vs. CTL (1.69 ± 0.11 vs. 1.07 ± 0.04, p < 0.05) and DMEC vs. CTLEC (1.29 ± 0.05 vs. 1.05 ± 0.03, p < 0.05) and significantly decreased in DMEC vs. DM, as seen in Fig. 3.

DISCUSSION

In this study, diabetic rats showed increased glycemia and NF-κB p65 levels, with increased GSSG and 3-NT; in addition, DM promoted a reduction in body weight and in Nrf2 levels, with the concomitant reduction of antioxidant defenses. EC proved to be effective in preventing weight loss in animals, and although it did not significantly reduce blood glucose, it was able to reduce important markers, such as NF-κB p65, iNOS and 3-NT, in addition to increasing the protein content of Nrf2 and CAT.

Before treatment with EC, the glycemia of diabetic groups was significantly elevated relative to their respective controls. However, EC showed a tendency to reduce these values from 4 weeks of treatment, remaining high after 8 weeks; contributing to lower weight loss of the

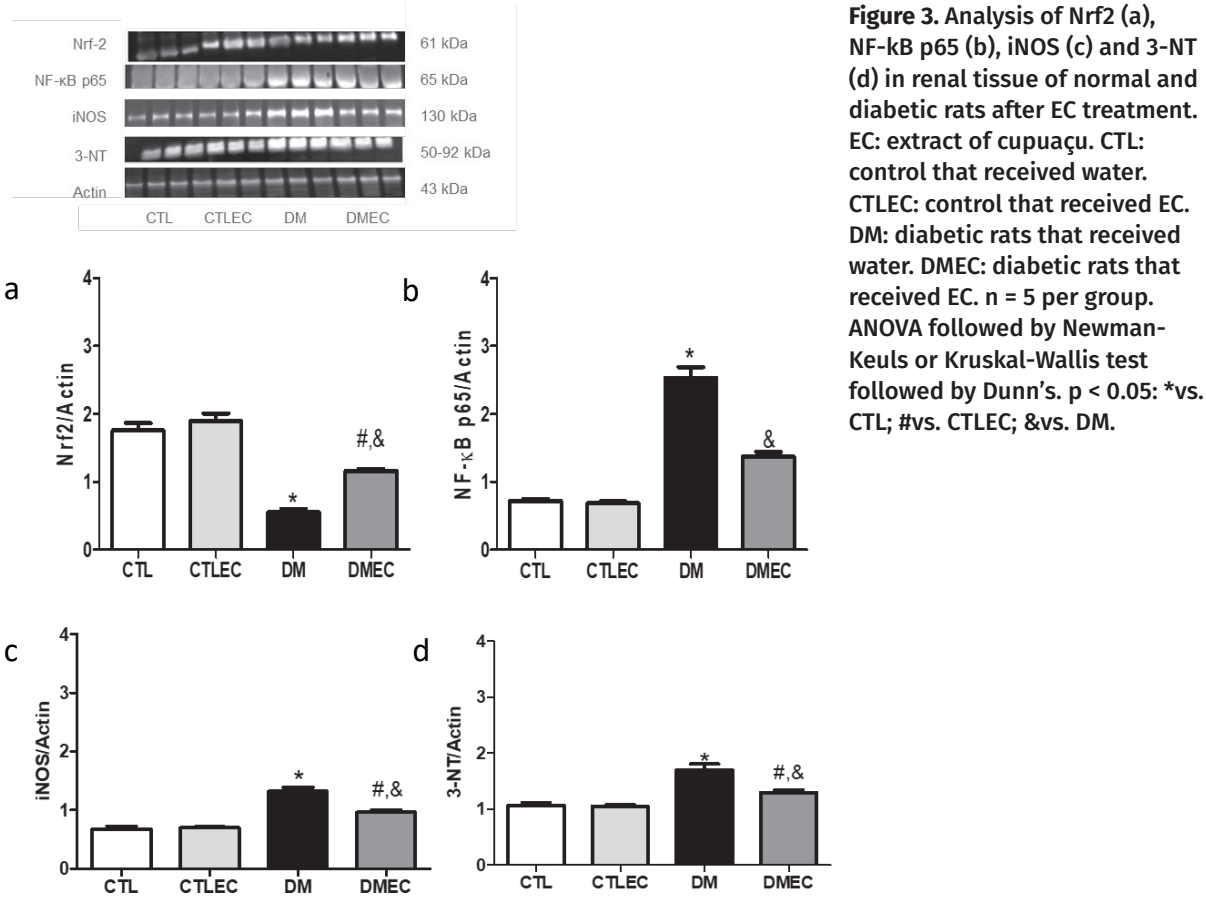

Figure 3. Analysis of Nrf2 (a), NF-kB p65 (b), iNOS (c) and 3-NT (d) in renal tissue of normal and diabetic rats after EC treatment. EC: extract of cupuaçu. CTL: control that received water. CTLEC: control that received EC. DM: diabetic rats that received water. DMEC: diabetic rats that received EC. n = 5 per group. ANOVA followed by Newman-Keuls or Kruskal-Wallis test followed by Dunn's. p < 0.05: *vs. CTL; #vs. CTLEC; &vs. DM.

DMEC group vs. DM, demonstrating a beneficial effect of this extract. Some studies have shown that intake of cupuaçu and Theobroma cacao liquors, fruit of the same cupuaçu family, promoted body weight gain in STZ rats, despite lower food intake, without affecting glycemic levels in STZ rats (Oliveira & Genovese 2013, Balisteiro et al. 2017), corroborating our findings. Another study with STZ rats demonstrated a reduction in glycemia and lipid profile after intake of cacao extract (Ruzaidi et al. 2005).

A study showed a significant reduction of SOD and CAT in renal tissue of diabetic rats compared to controls, whereas Maritim et al. (2003) did not find significant differences in SOD activity in diabetic groups for any tissue studied (Mestry et al. 2017, Maritim et al. 2003). Other studies demonstrated an increase in the GSSG/GSH ratio in patients with chronic kidney disease vs. control group (Puchades et al. 2009) and in 3-NT in rats with DN (Zou et al. 2017). These studies corroborate our data, in which GSSG and 3-NT were also increased and SOD-1, SOD-2 and CAT were decreased.

Another study evaluated the activities of SOD and CAT in several tissues of rats that received administration of cacao and showed that there was no significant difference in these activities in the spleen and liver, but there was in the thymus, in control group without cacao (Ramiro-Puig et al. 2007). In our study, the diabetic groups showed considerable attenuation in the renal expression of Nrf2 and enhanced NF-κB p65 vs. controls, demonstrating that EC was significantly able to reverse these values. A study that evaluated the kidneys of

STZ rats fed a high-fat diet also demonstrated a significant decrease in Nrf2 and an increase in NF-κB p65 vs. control rats (Selcuk et al. 2012), agreeing with our data.

A study performed with human hepatic cells treated with various glucose concentrations, showed reduced ROS and increased Nrf2 after treatment with cocoa and catechin (flavonoid extracted from cocoa); however, CAT levels remained unchanged. Cocoa alone was able to increase GPx and GR activities vs. control cells (Cordero-Herrera et al. 2015). Our data showed a significant increase in CAT and Nrf2 after EC, but SOD and GSSG did not present a significant difference. Until now, no literature study has demonstrated the effect of EC, specifically, on the oxidative stress generated by DN.

While our results showed a significant reduction in 3-NT, NF-κB p65 and iNOS after EC treatment, other studies revealed that treatment with proanthocyanidins of cocoa liquor inhibited the formation of 3-NT in the lungs of rats exposed to diesel exhaust particles, responsible for the formation of ROS (Yasuda et al. 2008), corroborating our data. Another study with macrophages also showed inhibition of NF-κB after treatment with polyphenol-enriched cocoa extract (Andujar et al. 2011). In addition to demonstrating an inhibitory effect on NF-κB, these compounds also acted in the degradation of IκB-α in the skin of mice treated with tetradecanoylphoric acetate, which contributed to inactivation of this factor (Lee et al. 2006).

Previous studies showed increased expression of iNOS in DM (Rodrigues et al. 2014, Nogueira et al. 2018, Punaro et al. 2014), agreeing with our findings. Cocoa of the cupuaçu family improved the antioxidant and anti-inflammatory potential by suppressing the expression of iNOS and COX-2 and the induction of SOD, CAT, GPx, GR and glutathione (GSH). It was demonstrated that the polyphenolic contents of cocoa, such as catechin, epicatechin and procyanidins, are activated and enhance the translocation of Nrf2 (Granado-Serrano et al. 2007, Cheng et al. 2013), confirming that this activation triggers even more cytoprotective genes that protect cells undergoing oxidative-mediated damage (Pandurangan et al. 2015).

Recent reports have shown that flavonoids, such as quercetin and rutine, exert protective action against apoptosis and inflammation through inhibition of iNOS/NF-κB and induction of Nrf2/heme oxygenase-1 pathways in cells and rats (Bahar et al. 2017). Rutine exhibited a protective role in iNOS suppression and activation of Nrf2 in stress conditions (Singh et al. 2019).The loss of Nrf2 activity increases the levels of nitrite and nitrate in the urine of diabetic mice induced by STZ (Yoh et al. 2008), suggesting critical contributions of this factor in complications of DM. In our study, we hypothesized that Nrf2 upregulation caused by cupuaçu flavonoids contributed to reduction of iNOS in renal tissue.

Another study showed that Nrf2 preserved pancreatic B cells in diabetic mice, through systemic glucose homeostasis in free radical-mediated damage (Yagishita et al. 2014). However, we hypothesized that the antioxidant effect of cupuaçu may decrease oxidative stress via Nrf2 due to an adaptive response in the endogenous antioxidant system that upregulates the Nrf2-mediated response. In healthy cells, the Nrf2 and NF-κB regulation are coordinated with the aim of maintaining redox homeostasis; however, this regulation is disturbed in pathological conditions favoring a therapeutic intervention (Ganesh et al. 2013). Some molecular studies have demonstrated the involvement of both transcriptional regulators (NF-κB and Nrf2) in the pathophysiology of several diseases (Negi et al. 2011). Research demonstrated the protective effect of allisartan by inhibiting NF

κB and activating the Nrf2 pathway in diabetic cardiomyopathy rats (Jin et al. 2021), revealing the crosstalk between these transcription factors. Another study using in STZ-induced diabetic mouse proved the antidiabetic effect of nanoceria (ceramic nanoparticles) by inhibiting NF-κB expression and increasing Nrf2, showing the modulation of Nrf2/NF-κB pathway (Khurana et al. 2018). Researcher showed ellagic acid diminished NF-κB and upregulated GSH, SOD and increased Nrf2, preventing oxidative stress, inflammation, apoptosis and renal damage, delaying DN (Altamini et al. 2022).

CONCLUSION

In conclusion, EC was able to restore the antioxidant profile, reducing nitrosative stress via Nrf2/NF-κB p65 and 3-NT in the kidneys of diabetic animals, in addition to reverting inflammation by decreasing iNOS and alleviating the loss of body weight, one of the consequences of the disease.

Despite not showing a significant reduction in blood glucose, EC could be used as a complementary therapy to improve the antioxidant profile and delay the progression of diabetic complications in tissues, such as kidney. However, further studies are needed to better elucidate the mechanisms and activity of cupuaçu in diabetes.

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Daniela B. B. Rodrigues, main author, responsible for the execution and development of all study. Giovana Rita Punaro contributed with the realization of laboratorial experiments, review, data processing and reading of the manuscript. Deyse Yorgos Lima contributed to the implementation of the protocol with animals, as well as subsequent experiments. She assisted in revising the manuscript. Adelson Marçal Rodrigues contributed to the implementation of the protocol with animals. Samuel Pugliero contributed to the development of the research and execution of the protocol with animals, as well as subsequent experiments. Elisa Mieko Suemitsu Higa worked as advisor and supervisor, responsible for the research financing and coordination.